DECLARATION OF JOSEPH FISHER UNDER 37 C.F.R. §1.131	Application Number	09/293,670
	Confirmation Number	5176
	Filing Date	April 16, 1999
	First Named Inventor	Joseph Fisher
	Examiner	Teresa Wessendorf
	Group Art	1639
	Attorney Docket No.	RIGL-036CIP

This Declaration with the attached Exhibits are being submitted in conjunction with the Applicants' Response to the Office Action dated February 24, 2006.

I, Joseph Fisher, M.D. Ph.D. do hereby declare as follows.

1. I am listed as an inventor of the above-referenced patent application.

2. Between June and September, 1997, I was a Scientist at Rigel Pharmaceuticals, Inc. (hereinafter "Rigel"). During this time, I was part of a program focused on the discovery of intracellularly-active peptides. The strategy employed by this program involved infecting cells with a library of retroviral vectors encoding candidate peptides, and selecting cells with an altered phenotype using fluorescence activated cell sorting (FACS)-based methods. The idea of using more than five FACS parameters to identify retrovirally-delivered, intracellularly-active peptides was developed before July 31, 1997.

3. I understand that the claimed subject matter of the above-referenced patent application relates to screening methods that include sorting a population of retrovirally infected cells using at least five fluorescence activated cell sorting (FACS) parameters. I

have been asked to provide factual evidence relating to my activities at Rigel with respect to the claimed subject matter before and after July 31, 1997.

4. Experiments confirming the applicability of FACS-based screening methods that employ at least five FACS parameters to the discovery of retrovirally-delivered intracellularly-active bioactive peptides were performed prior to July 31, 1997.

5. Exhibit A, which is a copy of pages 24 and 25 of my laboratory notebook, describes the results of an experiment in which cells were treated to induce exocytosis, and sorted using five FACS parameters. Exhibit A is dated prior to July 31, 1997. The top four graphs of page 25 show FACS results obtained from DMSO-treated cells (control), and the bottom four graphs of page 25 show FACS results obtained from A23187-treated cells (experimental). The top left graph of each group of four graphs shows results obtained from the parameter used to detect FM143, a fluorescent dye. The top right graph of each group of four graphs shows results obtained from the parameter used to detect FITC, another fluorescent dye. The bottom left graph shows results obtained from the parameter used to detect propidium iodide. The bottom right graph shows results obtained from parameter used to detect front light scatter as well as, independently, the parameter used to detect side light scatter. Thus, Exhibit A demonstrates the applicability of FACS methods that employ at least five FACS parameters to the discovery of retrovirally-delivered intracellularly-active bioactive peptides, before July 31, 1997.

6. Exhibit B, which is a copy of pages 112 to 120 of my laboratory notebook, describes an experiment in which and MC9 and CEM cells are transfected with a library of retroviral vectors that encode peptides. Exhibit B demonstrates that CEM and MC9 cells were transfected with a library of retroviral vectors between August 22 and August 27, 1997.

7. In September 1997, a method that included infecting cells with a library of retroviral vectors encoding candidate bioactive peptides, and selecting cells with an altered phenotype using five fluorescence FACS parameters was reduced to practice.

8. Exhibit C, which is a copy of pages 138 and 139 my laboratory notebook, describes an experiment in which retroviral vector library-infected cells are stimulated staurosporine to induce apoptosis, and sorted using five FACS parameters: side scatter ("ssc"), front light scatter ("fsc"), and three separate fluorescence parameters: ("fl1", "fl2" and "fl3"). Results for control cells not contacted with staurosporine are shown in the graphs on the left hand side of page 139, and results for experimental staurosporine-treated cells are shown in the graphs of the right hand side of page 139. Thus, Exhibit C demonstrates reduction to practice of a method that includes infecting cells with a library of retroviral vectors encoding candidate bioactive peptides, and selecting cells with an altered phenotype using five FACS parameters, on September 8, 1997.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Respectfully submitted,

Date: June 25, 2006

Joseph Fisher, M.D. Ph.D.,

Attachments: Exhibits A - C

EXHIBIT A

HMC-1 - Exocytosis Trace Dyes

- TM, FM-143 and ConA FITC as exocytosis Traces on HMC-1 Cells.
- HMC-1 Cells - From Alexis Spinner ~ 10^6 cells/ml, Highly Viable
- Spin/Wash 5×10^6 Cells in MT
- Divide into 2- ½ Incubate in MT 10' 37°C
  " " " + Succinyl ConA 100µg/ml } 37°C 10'
- Wash SOA Cells 2x MT
- Take up Cells in 1ml MT (no BSA) in 4 Tubes
  - A) DMSO } + FM-1-43 - 2.5µg/ml ⇒ 37°C 10'
  - B) + AZ3187 1µg/ml }
  - C) DMSO } + Con-A-FITC 25µg/ml ⇒ "
  - D) AZ3187 " }

Wash Cells 1x in MT - Take up in 1MT for FACS

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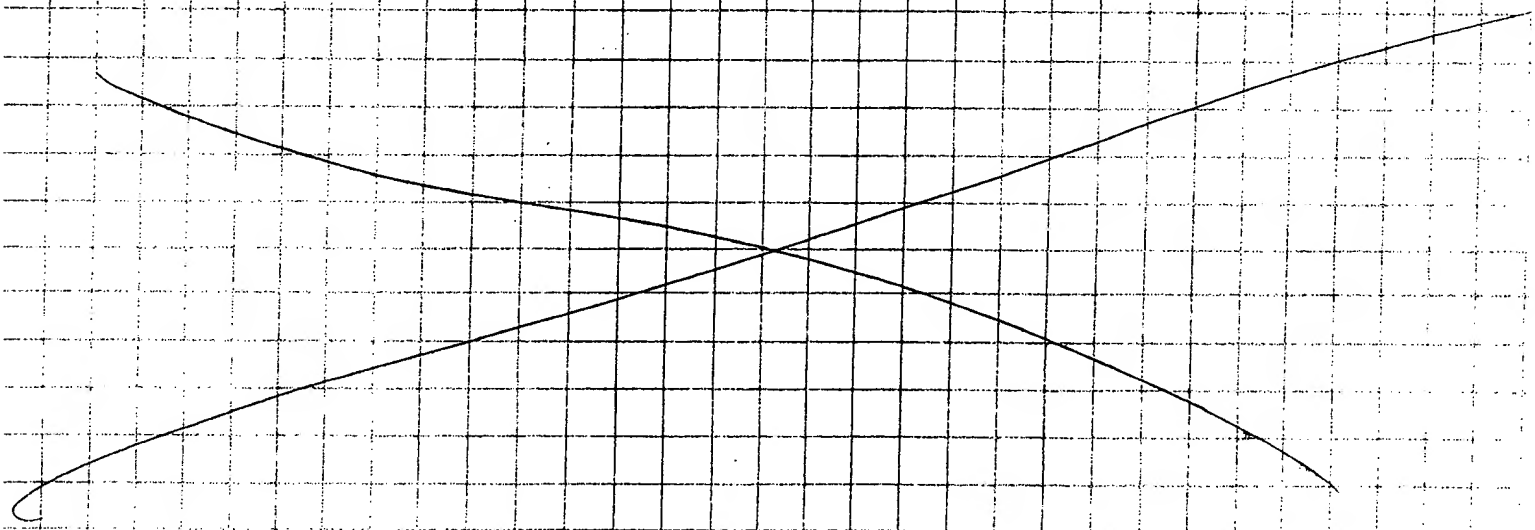
2 } D
3 } A
4 } B



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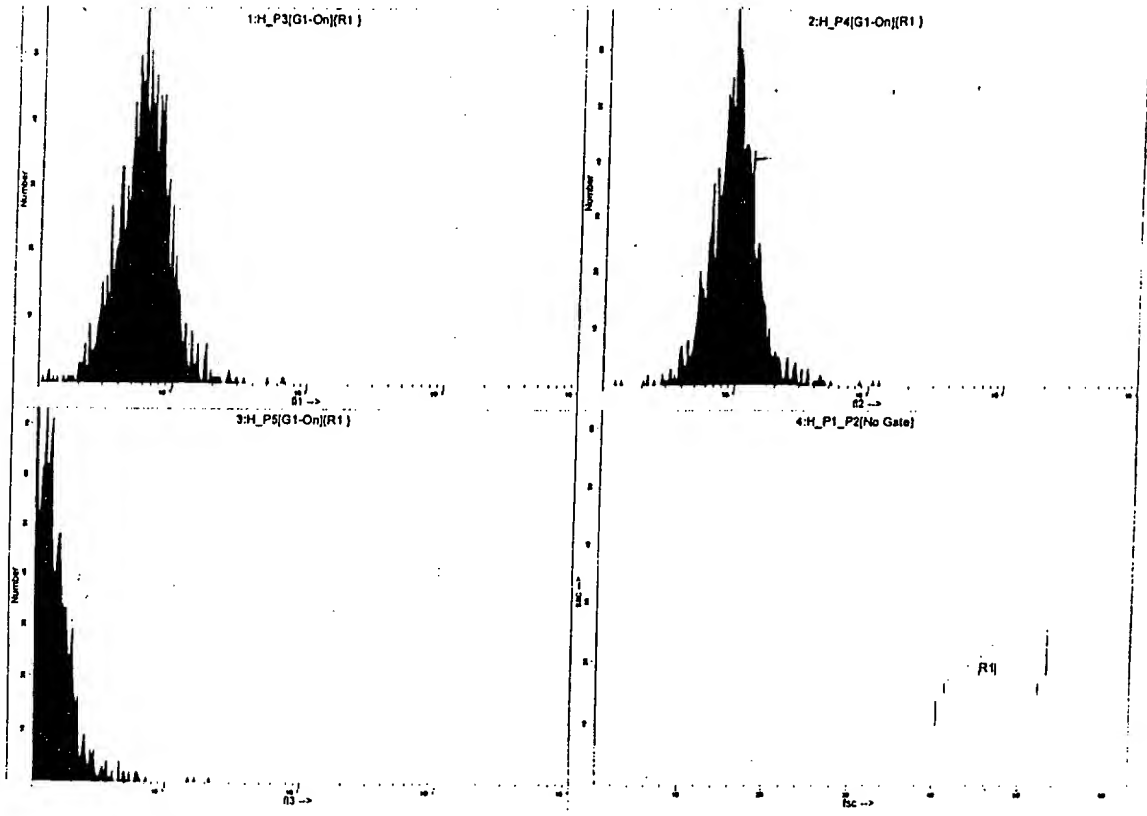
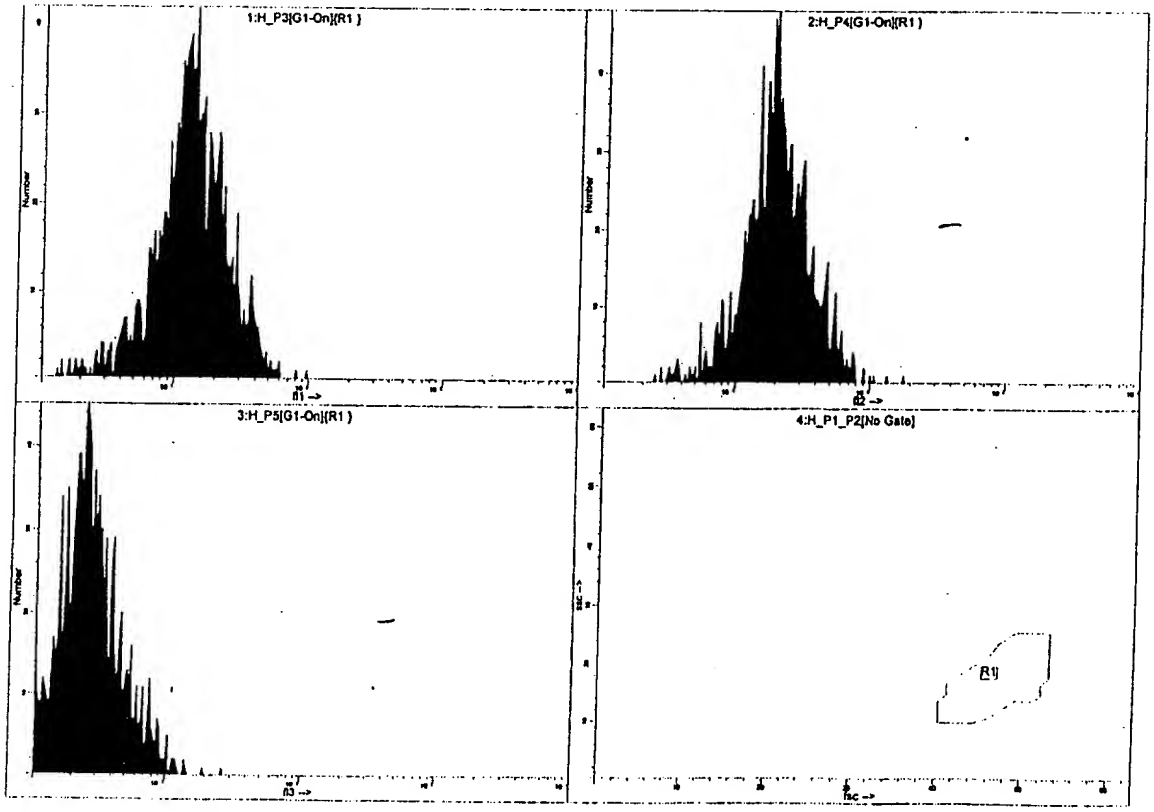


DMSO 10'
37°C



1ug/ml
A23187 10'
37°C

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James Joern

Date

Invented by
Jack Fisk

Recorded by

Date

8/22/97

EXHIBIT B

Phoenix E Cell Transfection ⇒ For MC9 cell Infection

- Use Susans Protocol (x2) So 2 wells of 6 well Plate / Transfection
- DNA - From Jenny Wang

			Randy's Nomenclature	Jim's Nomenclature	
1	(10µg) =	6.6λ			Rab3a and Synaptotagmin
2		6.3λ			Constructs
3		8.9λ			
4		9.1λ			
5	- New IRes Hook		43-13	129.13	10µg = 11.6λ
6	- " " 6FP		010.25	010-25	10µg = 11.1λ

From Jim L

- Follow Susans Protocol - Put Precipitate / Chloroquine on cells at 11AM
- Nice "Peppery" Precipitate Seen on all Transfectants

Protocol on next Page.

7PM ⇒ Aspirate DNA
- Wash 1x in Phoenix Media
- Add 2ml / well Fresh Media

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Protocol for transfection of Phoenix cells and infection of nonadherent target cells

Day 1:
seed Phoenix cells (Es or As) in 6 well plates at $8x10^5$cells in 1.5 ml (DMEM + 10% FBS + P/S) per well

Day 2: $CaPO_4$ Transfection

2wells

per well:

5ug DNA	10ug DNA
30.5ul 2M $CaCl_2$	61λ 2M CaCl2
219ul H_2O	438λ H_2O
250ul 2X HBS	500λ 2X HBS

allow all reagents to come to room temperature 30mins. before starting (do not warm up in H_2O bath)

add 50mM chloroquine at 2ul/well (50um final)

mix $CaPO_4$ reagents in 15ml polypropylene tube:
pipet 5ug DNA to side of tube
pipet 30.5ul of 2M $CaCL_2$ away from the DNA
mix the two together with the addition of 219ul of miliQ H_2O
then using a 1ml pipet, add 250ul of 2X HBS and quickly bubble air through the pipet for 2 to 10 secs. (the time is 2
HBS batch dependent)
immediately add mixture dropwise to well
microscopically visible precipitate should appear within a few minutes

incubate 8hrs
remove medium, wash once, and replace with 1.5ml medium

Day3:
move transfected plates to 32^0C

Day 4: Infection of target cells
collect virus supernatent from transfected wells (1.5 ml) into 15 ml tubes and add either 1.5ul of 5mg/ml polybrene
or 1.5ul 5mg/ml protamine sulfate
cfg out cells and debris at 2500 RPM for 5 mins. or alternatively, filter through .45um acrodisc syringe filter
count target cells and distribute $5x10^5$ cells per virus supe to 15ml tubes and pellet 5 mins. 2500 RPM
resuspend each pellet of target cells with virus supe and transfer to one well of a 24 well plate
seal plate with parafilm and cfg at RT for 90 mins. at 2500 RPM
Remove parafilm and incubate plate over night at 32^0C

Day 5:
collect and pellet each well of target cells and resuspend in 4ml and transfer each to a 6cm plate

Day 7 or Day 8:
at 48 to 72 hrs. post infection target cells are ready to analyze for expression

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8/23/97

- Transfections of ϕE Cells - (Cont.)
- This morning. 24 hrs post Transfection Start
  Look at Cells by Fluorescence.
  GFP ⊕ Cells Seen in #3, 4, and 6
  3 and 4 must be CTIG Vector (inducible with Ires GFP)
  1 and 2 " be pop Hook vector.
- Remove old Media
- Add 2ml/well of Warmed MC9 Media - 12AM

MC9 Positive Control Peptides

MC9 Cells - WT

Scaffold Hook	~75% Hook+	From Amy:
Synaptotagmin	~50% "	
RAB	- " "	

- Resuspend 2ml Cells, Take up in .3ml MT
  100λ/Tube
  ⇒ one gets FM143 1μM + 2μM Ionomycin } 37°C ⇒ 30'
  " " " " " "
  " " PI

View in FACSCAN

- 001	WT	5 -	WT
- 2	Hook	6 +	WT
- 3	Synaptotagmin	7 -	Hook
- 4	RAB	8 +	"
		9 -	Synaptotagmin
		10 +	"
		11 -	RAB
		12 +	"

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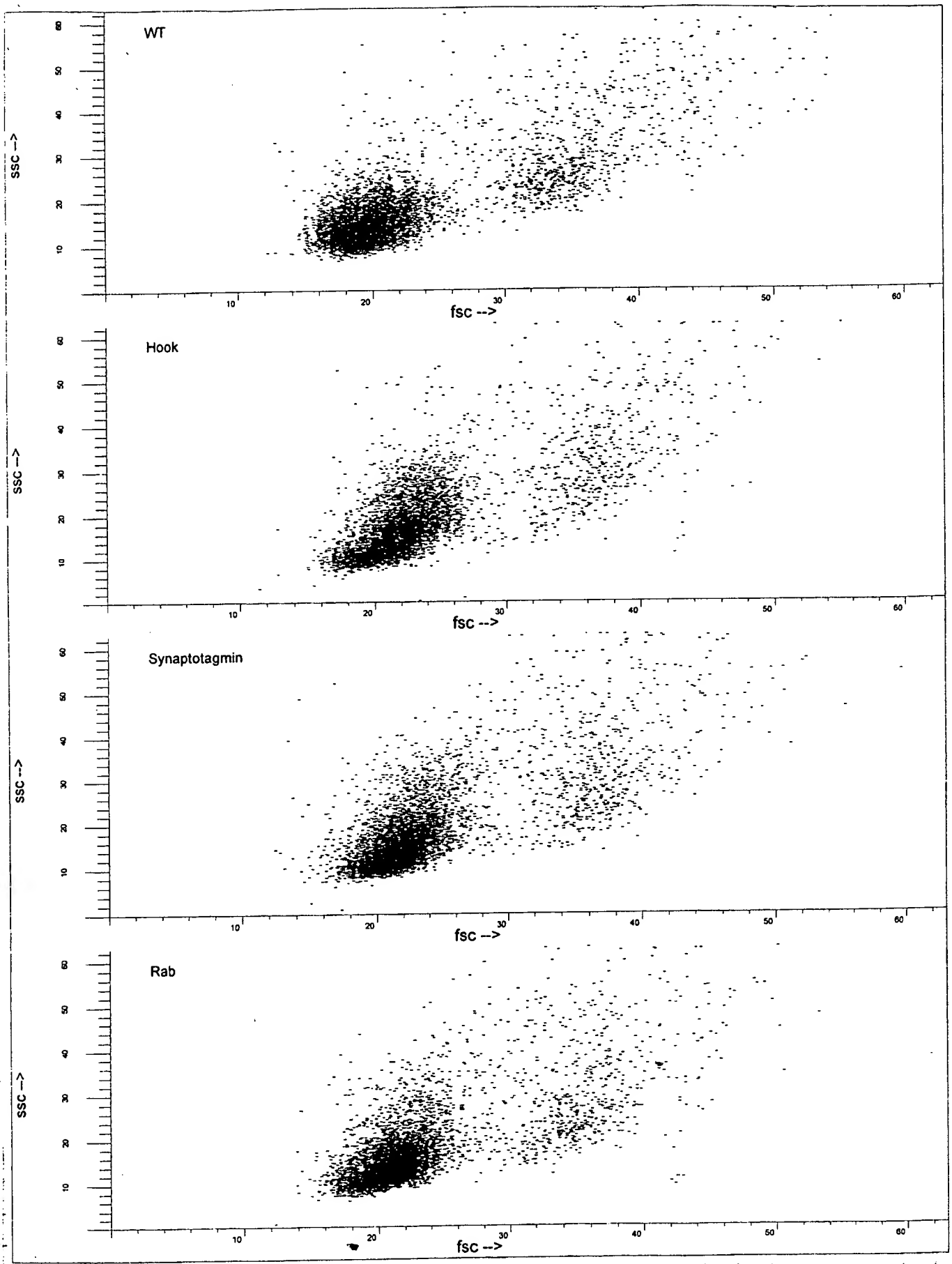

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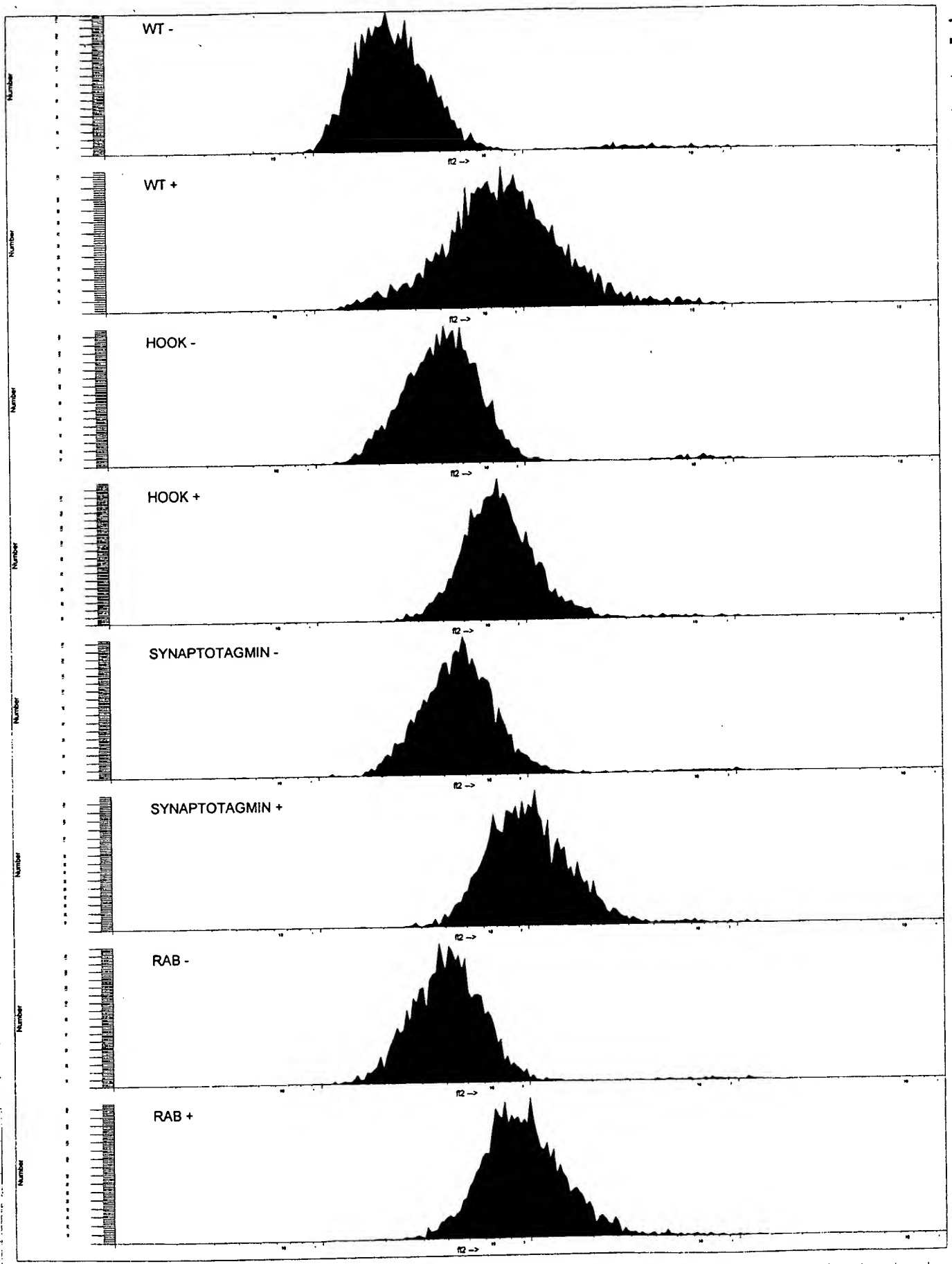

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8/25

= MC9 Cell Infection (Cont)

- Wells 3/4 + 6 of Transfection Look Significantly Brighter for GFP Than they did on 8/23
- ~1PM - Remove Viral Supe - Spin at 2500 RPM x 15' RT
- MC9 Cells, ~2.5×10^6/ml
- Spin Down 2ml x 6 MC9 cells (~5×10^6/Tube)
- Add Viral Supes
- Divide Each into 2 wells of a 6 well plate (~2ml / 2.5×10^6 Cells/well)
  Add 4λ of 5mg/ml Polyamine Sulfate / well so FC = 10μg/ml
- Seal Plates and Spin for 90' at 2500 RPM
- Culture ON at 37°C (~3:30 PM →)

- MC9 Cell Harvest - For Future cDNA Library Construction

Cells ~ 2×10^6/ml
- Spin down 200ml Cells
Wash 2x in Cold PBS / Aspirate
- Freeze on Dry Ice - 2 tubes x 2×10^5 Cells/Tube
- Store at -80°C

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8/24/97

MC9 Infections (Cont.)

- ~~2 x 6 well~~ Plates infected Yesterday
- ~ 11AM, Take Cells out of Wells/Pool, Wash Cells with 2ml MC9 Media, Spin, Decant
- Take up Pellets 1→6 with 12ml MC9 Media and Plate in T.75s
- ⇒ Quick Look at #6 Showed some GFP (+) Cells.

ires GFP Library Inf. Transfection

- Susan Plated 20 60 mm Plates of ΦE Cells Yesterday, today ~ 40% Confluent
- Randy Supplied DNA 10-62 Library - 14me, ires GFP 850 µg/ml
- For Each 60mm Plate add (Plates have 6ml of Media)

8λ Chloroquine (50mM)
10µg DNA (11.8λ)
122λ $CaCl_2$
876λ H_2O
1ml 2x HBS

Follow Standard Procedure

Transfected 17 Plates From 11:30AM → ~12:30AM

~ 6:30PM
- Aspirate Media
- Wash Cells 1x in PBS + Catt
- Add Warm MC9 Media - ~~8 ml~~/Plate

⇒ 37°C ~ 7PM

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CEM - Library Infection

Library in resCFP, ~10^6 Complexity of random 14-mer Peptides - Part of 2nd IgE Screen Library.

- Yesterday Susan S. Put CEM Media on Library Infected ΦA Cells (After She Harvested her virus ~3PM) - Today Renae Spuns (~4PM) Spin at 2500 RPM x 10' Add PS to 10μg/ml
- CEM Cells, ~1.1×10^6/ml
  Spin Down 60ml (~ 6.6×10^7 Cells Total)
  - Divide Pellets into 8 x 12ml Supers ⇒ / 8 T-75s ⇒ 8.25×10^6 Cells/Flask
    Spin T-75s at 2500 RPM
    4:45 → 6:15
    Take out and Put at 37°C ON

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8/27/96

<ΦE Library Transfection>

After GFP⊕ Cells Seen Today, but a minority
~4PM Transfer Cells to 32°C

- Split MC9 Cells for tomorrow's Infection

CEM Cells - Library Infection

~1PM (22 hrs Post Infection Spin) Spin All infected CEM Cells - Decant Supe
Take up in 90ml Fresh Media
Plate in 3 x T-150's

- Take out 1ml of Library Infection, 1ml of WT Cells
Annexin - PE / PI Stain as done on 8/13 (use those Controls As well)
View in FACSCAN

Files 001 WT
002 Library Infected

⇒ See next page
Some GFP⊕ Cells Staying up in Library Infected After 22 hrs

Make MC9 Media

DMEM (has Pyruvate and Glutamine)
18mg/500ml Asparagine
1X Non-essential AA
0.05mM 2ME
PenStrep 1X
10% HI FBS
10% T-Stim Conditioned Media

.2um Sterile Filter

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9/8/97

EXHIBIT C

CEM - Libray Infected - Apoptosis Induction

XX gae me Libray Infected Cells to test DNA Rescue Methods - CEM ~ 2.4×10^6/ml
Take 8ml (2×10^7 Cells) + 4ml Fresh Media, Bring to 1μM Staurosporine
→ 37°C 10AM → 4PM (6 Hours)
→ Annexin PE Stain as usual Procedure = files

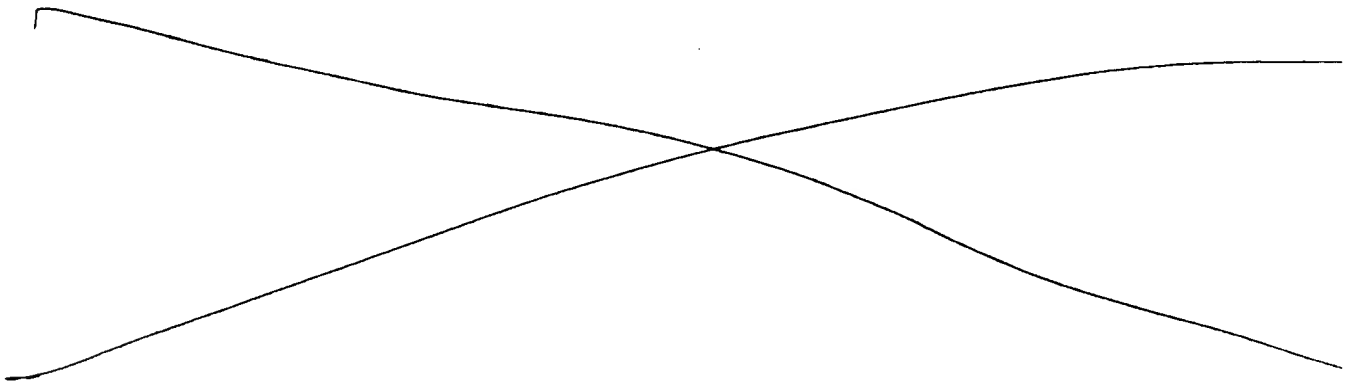
.015 GFP ONLY
16 Annexin PE ONLY
17 PI ONLY
18 GFP Libray - ϕ Stavo
19 " " + Stavo 7hrs
} Nav Settings

CEM - Libray - Stavosporine treatment 2x (9/3) - Nav 5 days post treatment

Take .5ml of Culture - Add PI
- FACSCAN - .001 - Libray untreated
.002 Treated Stavo 2x

MC9 Libray - GFP Enriched

- GFP Enriched Cells From Last week - nav ~ 2.8×10^6/ml x 100ml
- Split Back to ~ 10^6/ml for Tomorrow's Sort
- Remainder of Cells, ~ 2×10^8 cells
Spin / Decant, Freeze in 5 vials (4×10^7/vial) at -80°C)



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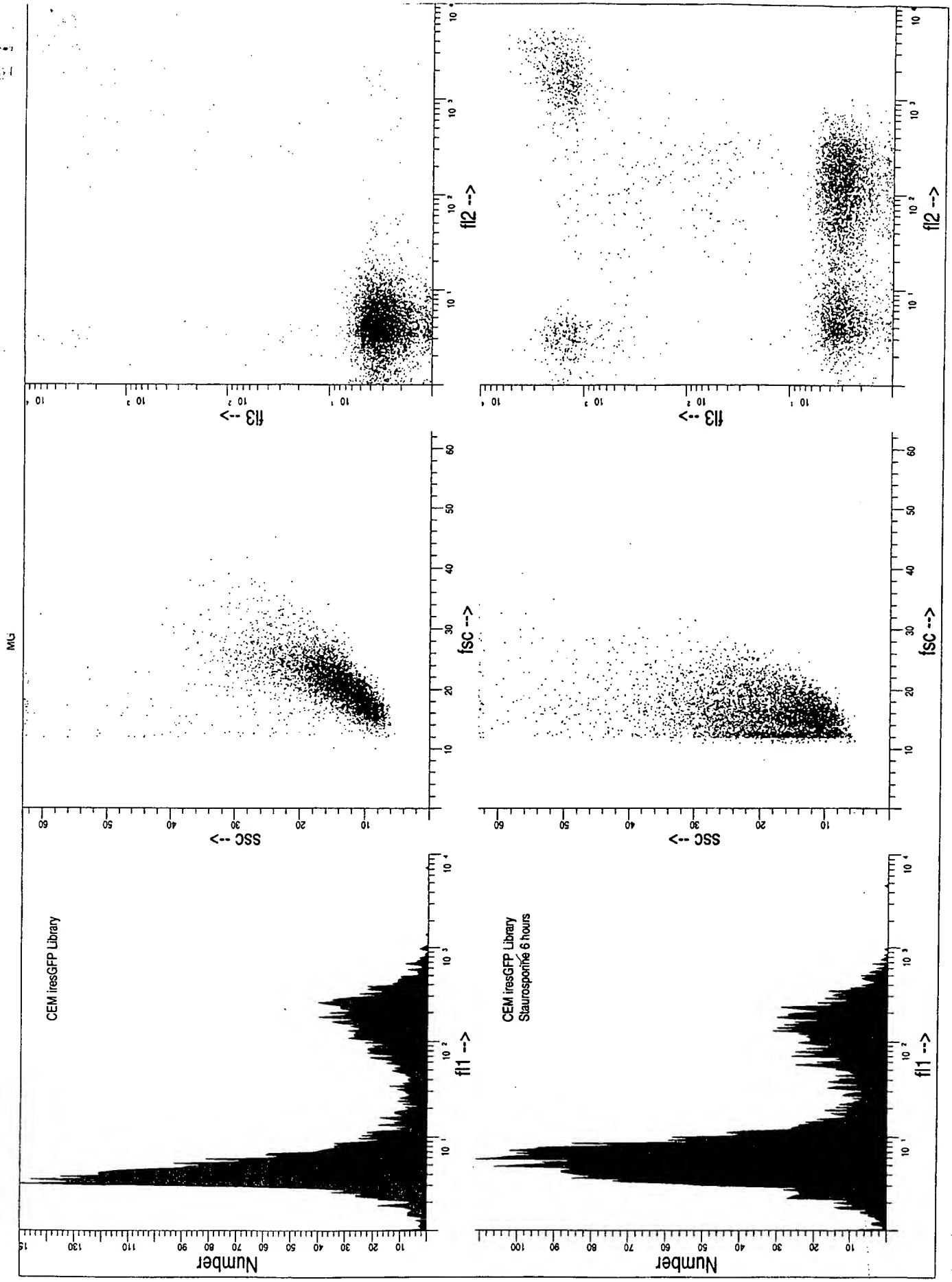

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